(51) International Patent Classification ^{6} : C12Q 1/48, 1/70, 1/68	A1	(11) International Publication Number: **WO 96/23076**
		(43) International Publication Date: 1 August 1996 (01.08.96)

(21) International Application Number: PCT/US96/01257

(22) International Filing Date: 26 January 1996 (26.01.96)

(30) Priority Data:
08/379,851   27 January 1995 (27.01.95)   US

(71) Applicant: THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DE-PARTMENT OF HEALTH AND HUMAN SERVICES {US/US}; National Institutes of Health, Office of Technology Transfer, Box OTT, Bethesda, MD 20892-9902 (US).

(72) Inventors: HENEINE, Walid; 2001 Hollidon Road, Decatur, GA 30333 (US). FOLKS, Thomas, M.; 3815 Belle Glade Trail, Lithonia, GA 30058 (US). SWITZER, William, Marshall; 820 Stephenson Ridge, Stone Mountain, GA 30087 (US). YAMAMOTO, Shinji; 3131 North Druid Hills Road, Decatur, GA 30033 (US).

(74) Agents: NEEDLE, William, H. et al.; Needle & Rosenberg, The Candler Building, Suite 1200, 127 Peachtree Street, N.E., Atlanta, GA 30303-1811 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS FOR SENSITIVE DETECTION OF REVERSE TRANSCRIPTASE

(57) Abstract

The present invention provides a method for detecting the presence of a retrovirus in a biological sample comprising the steps of: a) contacting the biological sample with an RNA template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample; b) amplifying the synthesized DNA; and c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

Applicants: Bernard Conrad, et al.
U.S. Serial No. : 09/490,700
Filed: January 24, 2000   **Exhibit 5**

METHODS FOR SENSITIVE DETECTION
OF REVERSE TRANSCRIPTASE

5

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

10

The present invention provides a method for detecting the presence of a

retrovirus in a biological sample by detecting the presence of the enzyme, reverse

transcriptase. The method utilizes a template and primers in conditions under which a

DNA strand is synthesized and subsequently amplified by the polymerase chain reaction,

15   if reverse transcriptase is present.

BACKGROUND ART

Retroviruses are widely distributed in vertebrates and are known to cause a

20   variety of diseases in man and animals including immunodeficiencies, leukemias and

lymphomas (1). The entire retrovirus family is characterized by the presence of a unique

enzyme, reverse transcriptase (RT), which transcribes the viral genomic RNA into a

double-stranded DNA copy (1). This feature has led to studies of the unique enzymatic

function of RT for two main applications. First, the presence of RT has been the basis

25   for diagnosis of retroviral infection and for the generic detection of the presence of

retroviruses in cell cultures and in the infected host. Second, the RT enzyme constitutes

a primary target for antiviral drug intervention (1,2).

2

The polymerase function of RT from several retroviruses has been well characterized and shown to require an RNA template, a primer, triphosphate, a divalent cation and physiological salts (1). Therefore, assays for RT activity have conventionally used these reagents and conditions to measure the ability of a sample to produce a DNA

5   copy of a known exogenous RNA template (e.g., poly rA) by synthesizing a complementary DNA oligonucleotide primer with radiolabeled nucleotides (e.g., ^{3}H- or ^{32}P-dTTP). RT synthesized DNA has been detected by measuring incorporation of the labeled nucleotide (3,4) and more recently, by assays which employ nonradioactive nucleotides in enzyme linked immunosorbent assay (ELISA) formats have been

10   developed (5-8) and by polymerase chain reaction (PCR) (17), as further described below.

Although RT assays continue to be an essential laboratory tool for the identification of known and novel retroviruses (4-11), the successful use of RT assays

15   has been limited to the detection of retroviral particles in culture supernatant (4-8), which has the disadvantage of requiring that a virus be cultured before detection. By the present methods, lentiviruses, such as human immunodeficiency virus-1 (HIV-1) may be readily detected, but oncoviruses, such as human T lymphocytic virus types I and II (HTLV-I and HTLV-II), are much more difficult to detect, presumably because of poor

20   RT activity and because they are typically cell-associated, which means their RT is less accessible for detection in culture supernatants (6-8). This limitation has rendered RT testing of little value in the detection of HTLV infection.

Despite multiple attempts to improve the sensitivity of RT assays, the direct detection of retroviruses in clinical samples (e.g., serum) has been unsuccessful (4-12). For example, in studies of HIV-1 infected individuals, detection of virus in plasma by RT assays has been largely abandoned because of the low sensitivity of this method (12).

5

The inability to detect RT activity in serum hinders the use of this virological marker in diagnosing disease, monitoring drug efficacy in patients, monitoring virus load and predicting disease progression. Present methods for qualitative and quantitative detection of plasma HIV-1 include viral isolation and p24 antigen capture

10 (13-15). However, these assays have disadvantages. Virus isolation from plasma requires virus culture that is typically maintained for 14 to 28 days and is labor intensive, time consuming, fraught with biological variation and is not a universal marker in the infected population given the low levels of HIV-1 in patients with CD4+ T lymphocyte counts of >200/mm³. Similarly, p24 antigen, either free, virion-associated, or immune

15 complexed, is not always present in the HIV-1 infected population.

RT-PCR permits the qualitative detection of the cell-free virus in plasma using an exogenous RT and a primer pair of known sequence to amplify viral RNA sequences in the plasma (15,16). Although RT-PCR has been reported to be highly sensitive, this

20 assay requires RNA extraction and multiple sample manipulations that may increase the risks of PCR contamination. The RT-PCR assay may be complicated further by the lack of a standardized universal quantitative test and the variabilities that may be incurred during processing and storage with regard to the degradation of the genomic RNA. Although RT-PCR, like antigen capture, is highly specific, a knowledge of the

4

nucleotide sequence of the target retrovirus fragment is necessary for primer

development. Given this limitation, RT-PCR is not suitable for detecting variant, novel,

or unknown retroviruses.

5   Recently, two assays for RT, which use the RNA of bacteriophage MS2 or

brome mosaic virus (BMV) as a template and PCR as a detection system, have been

reported to be highly sensitive for the detection of murine leukemia virus RT and other

stocks of retroviruses in serum (18,19). However, the evaluation of the specificity and

the sensitivity of these assays on adequate numbers of serum specimens was not

10   disclosed (18,19). In addition, in both of these assays, problems with inhibitors of RT

activity in serum and nonspecific background RT activity have been described (20),

raising serious questions about the diagnostic value of these assays.

        This invention provides a RT assay that employs a PCR-based amplification

15   system to detect a known cDNA product of the RT reaction. The assay of the present

invention, referred to hereafter as Amp-RT, is highly sensitive and specific, requires no

knowledge of viral genomic sequence and allows the detection of RT activity in

samples of individuals infected with retroviruses or any other biological entity that

produces RT.

20

SUMMARY OF THE INVENTION

        The present invention provides a method for detecting the presence of a

retrovirus in a biological sample comprising the steps of: a) contacting the biological

sample with an RNA template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample; b) amplifying the synthesized DNA; and c) detecting the amplification of the
5 synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

The present invention also provides a method of detecting the presence of a
10 retrovirus in a biological sample comprising the steps of: a) contacting the biological sample with an RNA template, wherein the RNA template is a suitable region of the encephalomyocarditis virus which consists of the ribonucleotide of SEQ ID NO:4 and a complementary DNA primer, wherein the primer is the oligonucleotide of SEQ ID NO:2, under conditions whereby the RNA template and the DNA primer will anneal and
15 a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample; b) amplifying the synthesized DNA, wherein the amplification is by the polymerase chain reaction method whereby the conditions for the amplification comprise 30-40 cycles of heating the synthesized DNA and a primer pair to 93 to 96°C for 30 seconds to one minute, 53 to 56°C for 30 seconds to one minute
20 and 70 to 74°C for 30 seconds to five minutes, wherein the primer pair consists of the oligonucleotide consisting essentially of SEQ ID NO:1 and the oligonucleotide consisting essentially of SEQ ID NO:2; and c) detecting the amplification of the synthesized DNA, wherein the detection of the amplification of the synthesized DNA is by Southern blot hybridization assay, with a probe consisting essentially of the

6

oligonucleotide of SEQ ID NO:3, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

5　　　　　　Additionally provided is a kit for detecting the presence of a retrovirus in a biological sample, comprising a suitable region of the encephalomyocarditis virus genome as an RNA template and a complementary DNA primer for reverse transcriptase and a primer pair for polymerase chain reaction, whereby each component is provided in separate containers or any combination of the components is provided in a single

10　container.

DETAILED DESCRIPTION OF THE INVENTION

As used in the specification and in the claims, "a" can mean one or more,

15　depending on the context in which it is used.

The present invention provides a method for detecting the presence of a retrovirus in a biological sample comprising the steps of: a) contacting the biological sample with an RNA template and a complementary DNA primer under conditions

20　whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample; b) amplifying the synthesized DNA; and c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of

reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus

in the biological sample.

5   The RNA template can comprise a suitable region of any ribonucleotide

sequence such as, for example, the encephalomyocarditis virus genome. As used herein,

a "suitable region" means a region of the RNA sequence having no significant secondary

structure, less than 50% G-C content and to which complementary DNA primers can be

generated which have T_m values within the range of reaction temperatures appropriate

for the synthesis of a DNA strand, as further described herein. The RNA template can

10   be of a length sufficient to produce a DNA product ranging in size from 100 to 500 base

pairs in length and most preferably about 300 base pairs in length. The RNA template

can be the ribonucleotide of SEQ ID NO:4.

As used herein, "complementary DNA primer" means an oligonucleotide which

15   anneals to the RNA template in a particular orientation to allow for the synthesis of a

nascent DNA strand in the presence of RT in the biological sample under the conditions

described herein.   Also as used herein, the "conditions" under which a DNA strand is

synthesized include the presence of nucleotides, cations and appropriate buffering agents

in amounts and at temperatures such that the RNA template and the DNA primer will

20   anneal and oligonucleotides will be incorporated into a synthesized DNA strand if

reverse transcriptase is present. More specifically, an example of these conditions is

provided in the Examples section. The described conditions have been optimized from

other known RT/cDNA synthesis protocols. It is generally known that other conditions

can be established for optimization of a particular RT reaction on the basis of protocols

well known to one of ordinary skill in the art. The DNA primer can be the reverse

primer of a primer pair to be used in a subsequent amplification by PCR, such as, for

example, the oligonucleotide of SEQ ID NO:2 (EMCR2).

5   The biological sample can comprise any biological tissue or body fluid (e.g.,

cells, serum, plasma, semen, urine, saliva, sputum, cerebrospinal fluid). The synthesized

DNA strand can be amplified by any of the amplification protocols known in the art now

or in the future, including but not limited to the polymerase chain reaction (PCR) (17),

the ligation amplification reaction (LAR) (21), the ligase-based amplification system

10 (LAS) (22), the self-sustained sequence replication (3SR) system (23), the transcription-

based amplification system (TAS) (24) and the Qβ replicase amplification method (25).

     For amplification of the synthesized DNA by PCR, the conditions for

amplification can include 30 to 40 (most preferably 35) cycles of heating the synthesized

15 DNA and a primer pair to 93° to 96°C (most preferably 95°C) for 30 seconds to one

minute (most preferably one minute), 53° to 56°C (most preferably 55°C) for 30

seconds to one minute (most preferably one minute) and 70° to 74°C (most preferably

72°C) for 30 seconds to five minutes (most preferably one minute).

20   As used herein, "a primer pair" refers to two primers, one having a forward

designation and the other having a reverse designation relative to their respective

orientations on a double-stranded DNA molecule which consists of a sense and antisense

sequence, such that under the amplification conditions described herein, the forward

primer anneals to and primes amplification of the sense sequence and the reverse primer

anneals to and primes amplification of the antisense sequence. Primers can be selected

for use in the amplification reaction on the basis of having less than 50% G-C content,

having minimal complementarity with other primers in the reaction (to minimize the

formation of primer dimers) and having T_m values within the range of reaction

5   temperatures appropriate for PCR. In addition, primers can be selected to anneal with

specific regions of the RNA template such that the resulting DNA amplification product

ranges in size from 100 to 500 base pairs in length and most preferably around 300 base

pairs in length. For example, in the conditions described above, the primer pair can

consist of the oligonucleotide of SEQ ID NO:1 (EMCF1) as the forward primer and the

10 oligonucleotide of SEQ ID NO:2 (EMCR2) as the reverse primer.

As used herein, "detecting" or "detection" of the amplified DNA refers to

quantitatively or qualitatively determining the presence of the amplified DNA strand

which is only synthesized if RT is present in the biological sample. The amplification of

15 the synthesized DNA can be detected by any method for the detection of DNA known in

the art. For example, detection of the amplified DNA can be by Southern blot

hybridization assay, by visualization of PCR products of specific molecular weight on

ethidium bromide stained agarose gels, by measurement of the incorporation of

radiolabeled nucleotides into the synthesized DNA strand by autoradiography or

20 scintillation measurement, by ELISA modified for the capture of a detectable moiety

bound to the amplified DNA, or any other detection method known to one of ordinary

skill in the art.

10

For detection by Southern blot hybridization assay, the synthesized DNA can be detected by a probe specific for the DNA synthesized from the template. For example, such a specific probe can consist essentially of the oligonucleotide of SEQ ID NO:3 (EMCP1). A Southern blot hybridization protocol for use in the invention is

5   demonstrated in the Examples.

The present invention provides a method for detecting the presence of a retrovirus in a biological sample comprising the steps of: a) contacting the biological sample with a suitable region of the encephalomyocarditis virus genome as an RNA

10   template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample; b) amplifying the synthesized DNA; and c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse

15 · transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

The present invention also provides a method of detecting the presence of a retrovirus in a biological sample comprising the steps of: a) contacting the biological

20   sample with an RNA template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample; b) amplifying the synthesized DNA by the polymerase chain reaction method whereby the conditions for the amplification comprise 30-40 cycles of heating the

synthesized DNA and a primer pair to 93 to 96°C for 30 seconds to one minute, 53 to 56°C for 30 seconds to one minute and 70 to 74°C for 30 seconds to five minutes; and c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological

5   sample, thus indicating the presence of a retrovirus in the biological sample.

The present invention further provides a method for detecting the presence of a retrovirus in a biological sample comprising the steps of: a) contacting the biological sample with a region of the encephalomyocarditis virus genome as an RNA template and

10   a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample; b) amplifying the synthesized DNA by the polymerase chain reaction method whereby the conditions for the amplification comprise 30-40 cycles of heating the synthesized DNA and a primer

15   pair to 93 to 96°C for 30 seconds to one minute, 53 to 56°C for 30 seconds to one minute and 70 to 74°C for 30 seconds to five minutes; and c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

20

As an example, the present invention can provide a method for detecting a retrovirus in a biological sample comprising the steps of: a) contacting the biological sample with a suitable region of the encephalomyocarditis virus genome as an RNA template, wherein the RNA template is the ribonucleotide of SEQ ID NO:4 and a

12

complementary DNA primer, wherein the primer is the oligonucleotide of SEQ ID

NO:2, under conditions whereby the RNA template and the DNA primer will anneal and

a DNA strand will be synthesized as an extension from the DNA primer if reverse

transcriptase is present in the sample; b) amplifying the synthesized DNA, wherein the

5   DNA is amplified by the polymerase chain reaction under conditions which comprise

about 35 cycles of heating the synthesized DNA and a primer pair to 95°C for one

minute, 55°C for one minute and 72°C for one minute, wherein the primer pair consists

of the oligonucleotide consisting essentially of the SEQ ID NO:1 and the

oligonucleotide consisting essentially of the SEQ ID NO:2; and c) detecting the

10   amplification of the synthesized DNA, wherein the amplification is detected by Southern

blot hybridization assay with a probe consisting essentially of the oligonucleotide of

SEQ ID NO:3.

Additionally provided is a kit for detecting the presence of a retrovirus in a

15   biological sample, comprising the enzymes, buffering agents, cations and

oligonucleotides well known in the art for carrying out RT and PCR reactions. The kit

also comprises a suitable region of the encephalomyocarditis virus genome as an RNA

template and a complementary DNA primer for reverse transcriptase and a primer pair

for polymerase chain reaction. The RNA template, the complementary DNA primer and

20   the primers of the primer pair can each be in separate containers or all or any

combination of these components can be combined in a single container. The

complementary DNA primer can be the oligonucleotide of SEQ ID NO:2 (EMCR2), the

RNA template can be the ribonucleotide of SEQ ID NO:4 and the primer pair can

13

consist of the oligonucleotide consisting essentially of SEQ ID NO:1 (EMCF1) and the oligonucleotide consisting essentially of SEQ ID NO:2 (EMCR2).

The present invention has additional applications based on the basic inventive principle that RT can be detected and quantitated in a biological sample. One application of this principle is the differential identification of retroviruses on the basis of the specific reactivity of the detected RT with antibodies against different retroviral RTs (for example, antibodies which can distinguish RT produced by HIV-1 or HIV-2 in infected individuals). Each retrovirus has a distinct RT, to which specific antibodies can be generated, thereby permitting identification of the specific retrovirus. The method of the present invention can be employed to first detect the presence of RT and various antibodies specific for RTs of different retroviruses can then be added to the biological sample to identify the type of retrovirus present. An antibody of known RT specificity will bind the RT present in the biological sample if the RT is produced by the virus to which the antibody is specific and will inhibit its activity, resulting in no DNA synthesis in the presence of antibody.

Another application of the present invention is screening patients for resistance to drug therapy. The susceptibility of RT to anti-RT drugs can be monitored over time in a patient receiving anti-RT drug therapy. The present invention can be employed to detect the emergence of anti-RT drug resistance in a patient by direct testing of the patient's serum for RT activity as an indicator of the susceptibility of the RT in the patient's serum to the anti-RT drug(s) used. If drug resistance is detected, alternative treatment strategies may be implemented. This invention obviates the need for the

14

lengthy and labor-intensive culture methods currently used to study drug resistance in patients.

A third application of the present invention is the monitoring of virus load in
5   patients infected with biological entities which produce RT. Quantitative measurement of RT over time can be correlated with survival and/or recovery rates in patients with illnesses caused by theses entities, for the purpose of following disease progression and prognosing the patient's illness.

10   The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

15

The following examples are intended to illustrate, but not limit, the invention. While the protocols described are typical of those that might be used, other procedures known to those skilled in the art may be alternatively employed.

20   **Viruses**

The retroviruses used in this study were prepared as follows. HIV-1 Lai and simian immunodeficiency virus (SIVB670) were propagated in peripheral blood lymphocytes (PBLs). Caprine arthritis encephalitis virus (CAEV-63) was propagated in fetal goat synovial membrane cells. HTLV-I and HTLV-II were obtained from

supernatants of MT-2 and Mo-T cell lines; simian retroviruses types 1 and 2 (SRV-1

and SRV-2) were grown in Raji cells; gibbon ape leukemia virus (GALV) was grown in

Jurkat cells and simian foamy virus type 3 (SFV-3) was grown in the Cf2Th cell line.

5   *In vitro* **transcription of the RNA template**

An RNA sequence from the encephalomyocarditis virus (EMCV) was used as

template and was generated from a plasmid vector obtained from Novagen (Madison,

WI, USA). A small EMCV sequence (350 bp) (SEQ ID NO:4) was amplified from the

plasmid, using standard PCR conditions and the primer pair T7-EMCF1 (SEQ ID NO:6)

10   and EMCR2 (SEQ ID NO:2). The sequence of T7-EMCF1 (SEQ ID NO:6) was:

5'GGTACCTAATACGACTCACTATAGGGAGACATTAGCCATTTCAACCCAT3'

and that of EMCR2 (SEQ ID NO:2) was:

5'GTTCATGACAGGCCGATACAGAGG3'.

15   To allow the *in vitro* transcription of this PCR product, the sense primer,

EMCF1: 5'CATTAGCCATTTCAACCCAT3' (SEQ ID NO:1), was modified at the 5'

end by adding a T7 promoter sequence: 5'GGTACCTAATACGACTCACTAT-3' (SEQ

ID NO:5).

20   The EMCV-amplified product was then transcribed with the large scale T7

transcription kit from Novagen according to the manufacturer's instructions. The DNA

in the RNA preparation was subsequently digested twice with 20 units of RNase-free

DNase (Promega) at 37°C for 60 minutes and the DNase was inactivated by heating at

95°C for ten minutes. The purity of the RNA preparation was checked for residual DNA

16

contamination by PCR amplification with EMCF1 (SEQ ID NO:1) and EMCR2 (SEQ ID NO:2) and subsequent Southern blot hybridization to the ^{32}P-end-labeled internal probe EMCP1 (SEQ ID NO:3): 5'TGCTCTCACCTTATCAAAATCCAAT3'.

5   **Amp-RT**

Twenty ul or less of the biological sample to be tested was added to 40 ul of RT buffer containing 10 ng of RNA template, 10 units of RNasin, 0.06% NP-40, 200 ng primer (EMCR2, SEQ ID NO:2), 0.8mM EGTA, 2mM DTT, 50mM Tris-HCl, 50mM KCl, and 10mM MgCl$_2$. The reaction was incubated at 37° C for two hours and then

10   heated at 95°C for ten minutes to inactivate any RT activity. A volume of 50 ul of standard PCR buffer (17) containing 0.5 units of Taq polymerase and 200 ng of the sense primer without the T7 sequence (EMCF1, SEQ ID NO:1) was added to this mixture. The reaction was cycled 35 times at 95°C for one minute, 55°C for one minute and 72°C for one minute. Twenty ul of the reaction product was electrophoresed in

15   1.8% agarose gels and Southern blot hybridized to a ^{32}P-end-labeled internal probe (EMCP1, SEQ ID NO:3) at 42°C overnight. The blots were washed in 2X SSC (NaCl and sodium citrate), 0.5% sodium dodecyl sulfate (SDS) for one to two hours. The blots were exposed from six to 24 hours.

20   To check the integrity and the purity of the RNA template preparation, RT reactions were carried out using the EMCV RNA template (SEQ ID NO:4) and an HIV-1 virus stock as the RT source. While the HIV-1 reactions were positive, control reactions which had no HIV-1, or which were pretreated with RNase before the addition of HIV-1, were all negative. These negative results indicated that the underlying

reaction of Amp-RT was RNA-dependent and that the RNA template and other assay

components were free of any contaminating target DNA. To further confirm that the

reaction was mediated by RT, Amp-RT reactions were prepared by using HIV-1 culture

supernatant in the presence of 2 ug of tetrahydroimidazo-benzodiazepin (TIBO)

5   compounds, which are non-nucleoside RT inhibitors (2). The results demonstrated

inhibition of the Amp-RT reaction only in the presence of the TIBO compounds, while

the DNA polymerase activity of Taq in the control amplification reactions was not

affected.

10   **Comparative sensitivity analysis of Amp-RT**

Many specific and generic assays are currently used to detect the presence of

retroviruses. To compare the relative sensitivities of these assays to Amp-RT, serial

10-fold dilutions of an HIV-1 culture supernatant were made in culture medium and

then divided into equal aliquots. Separate aliquots were subjected to testing by standard

15   RT assay, branched DNA detection, p24 antigen capture, 50% tissue culture infective

dose ($TCID_{50}$) determination, RT-PCR and Amp-RT.

Standard RT assay

RT activity was measured in culture supernatant with the template primer of

20   poly(rA).oligo dT according to the methods of Willey et al. (3). The enzymatic activity

was assessed by measuring the incorporated tritiated thymidine monophosphate.

Branched DNA (bDNA) detection of HIV-1

Branched DNA (bDNA) detection of HIV- 1 was performed according to the

manufacturer's instructions (Chiron, Emryville, California, USA). A cutoff of 5,000

RNA equivalents/ml was used, as recommended by the manufacturer.

5

p24 antigen capture

Levels of base dissociated-p24 antigens of HIV-1 were determined by a

commercially available ELISA kit from Organon Technika (North Carolina, USA).

10   **TCID$_{50}$ determination**

The TCID$_{50}$ of the HIV-1 viral stock was determined on PBLs as previously

described by McDougal et al. (26).

RT-PCR

15   Particle-associated HIV-1 genomic RNA in culture supernatant was extracted

with phenol/chloroform, precipitated with ethanol and reconstituted in 40 ul of RT

buffer [50mM Tris-Cl (pH 8.3), 20mM KCl, 10mM MgCl$_2$] (15). RNA was further

digested with 5 units of DNase-I (Promega) in the presence of 10mM sodium acetate at

37°C for 30 minutes. DNase was inactivated by heating at 95°C for ten minutes. For

20   reverse transcription, 10 ul of RNA was added to 20 ul of the RT mixture, containing

10mM DTT, 20 units RNasin, 0.875mM each of GTP, ATP, CTP and TTP, 200 ng of

reverse primer (SK39, SEQ ID NO:8) and 0.02 units of AMV-reverse transcriptase. The

mixture was reverse transcribed at 42°C for 45 minutes. Controls included 10 ul

aliquots that were not reverse transcribed. All samples were then amplified by PCR in

100 ul reaction volumes for 35 cycles, using SK38/SK39 (SEQ ID NOS:7 and 8) as

primers and the amplified products were Southern blot hybridized to the ^{32}P-labeled

SK19 (SEQ ID NO:9) probe (27).

5   The results, shown in Table 1, demonstrated that Amp-RT was the most

sensitive assay and was 100,000 times more sensitive than the standard RT assay,

10,000 times more sensitive than the bDNA detection system and the p24 antigen

capture assay and 100 times more sensitive than TCID$_{50}$ and RT-PCR. The p24

concentration in the undiluted HIV-1 sample was found to be 1.6 ng.

10

Testing of clinical samples with Amp-RT

        Forty-two serum samples from HIV-1 seropositive individuals enrolled in a study

of the natural history of HIV in homosexual men in Atlanta, Georgia, USA (28) were

tested by Amp-RT. The clinical stage of the HIV-1 infection in these men was

15   determined on the basis of the Centers for Disease Control and Prevention (CDC)

revised classification system (29). Laboratory markers, including CD4+ lymphocyte

counts and percentage of CD4+ lymphocytes in the blood, were determined by standard

flow cytometric methods. Serum samples from 20 healthy individuals who were

HTLV-I/II and HIV-1 seronegative were included as controls.

20

        Preliminary experiments using normal human serum spiked with HIV-1 were all

Amp-RT negative and indicated that preparation of serum samples for Amp-RT requires

special conditions different from those described for culture supernatant. For instance,

even small volumes (5-20 ul) of serum or plasma could not be directly tested by

20

Amp-RT because of protein precipitation that develops during the high temperature stage of the PCR cycle. Therefore, to avoid this problem, sera were ultracentrifuged and the Amp-RT assay was performed using the viral pellet.

5   For testing of culture supernatant, 20 ul or less was used for the RT reaction. For testing serum or plasma samples, 0.5 ml was diluted 10-fold in DEPC-treated phosphate buffered saline, clarified by centrifugation at 1,000 g for 10 minutes and then ultracentrifuged at 44,000 g for one hour. The pellet was suspended in 50 ul of RT buffer containing 0.6% NP-40 for 15 minutes and aliquots of 5-45 ul were used in the

10   Amp-RT assay.

The results of testing serum samples indicated that Amp-RT was able to detect RT activity in 36 (85.7%) serum samples (Table 2), 31 of which (86.1%) had detectable p24 antigen. This high sensitivity was also coupled with a high specificity since none of

15   the HIV-1/HTLV seronegative samples were positive. Of the six samples from patients classified as A1, three were Amp-RT positive (50%), while five of seven (71.4%) and 25 of 26 (96.1%) of the samples from patients classified as B2 and C3, respectively, were Amp-RT positive.

20   **Testing of the hepatitis B virus (HBV) by Amp-RT**

Because the polymerase gene of HBV has been reported to possess RT-like activity (30), Amp-RT was employed to detect the HBV-associated RT activity. Serum samples from 21 HBV-infected individuals were selected, including 11 samples with

detectable HBe antigen (Abbott HBe EIA, Chicago, USA) and ten other samples with no detectable HBe antigen. .

Despite the documented presence of HBV virions in 11 serum specimens, none

5   of the samples yielded positive results by Amp-RT analysis. These negative results exclude the possibility of the HBV-associated RT interfering in the Amp-RT assay, at least under these conditions, and therefore confirm the specificity range of the assay for the detection of only retroviral RTs.

10   **Detection of different retroviruses by Amp-RT.**

The ability of Amp-RT to detect a wide variety of retroviruses representing members of all three retroviral subfamilies was investigated. The lentiviruses tested included HIV-1, SIV and CAEV. HTLV-I, HTLV-II, GALV, SRV-1 and SRV-2 were representative of the oncoviruses and SFV-3 was representative of the spumaviruses.

15   Amp-RT was capable of detecting all retroviruses tested. Most importantly, viruses such as HTLV-I, HTLV-II and GALV, which are typically difficult to detect with the standard RT methodology, were easily identified with this assay.

These viruses could be detected by Amp-RT in dilutions containing 0.04 to

20   0.00004 ul of the original unconcentrated culture supernatant. The serial dilutions of the tested retroviruses were made in supernatant from uninfected cell lines (Hut-78, A301 or U937). All three supernatants consistently tested negative, as can be seen from the end point dilutions of the tested retroviruses.

22

Side by side comparison of previously published RT assays

To demonstrate that the assay of the present invention provides increased specificity and sensitivity in comparison to other published RT assays [22,23], a side by side comparison of these assays can be performed. These assays differ in the particular

5   RNA template sequence, primer sequences, RT buffer compositions, amplification conditions, detection conditions and sample preparation used. Therefore, for a comparison of the two assays, a panel of serum samples from HIV-1 seropositive individuals and from HIV-1 and HTLV-I/II negative individuals can be tested according to the teachings of the present assay and each other assay. Positive controls can include

10   normal human serum spiked with HIV-1 virus particles. The specificity and sensitivity of these assays can then be computed and compared.

Throughout this application various publications are referenced by numbers within parentheses. Full citations for these publications are as follows. The disclosures

15   of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Although the present process has been described with reference to specific

20   details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

●   ●

Table 1. Comparison of the sensitivity of Amp-RT in the detection of HIV-1 with other generic and virus-specific methods.

HIV-1 dilution	Std. RT	bDNA	p24 ag	$TCID_{50}$	RT-PCR	Amp-RT
10^0	+	+	+	+	+	+
10^{-1}	+	+	+	+	+	+
10^{-2}	+	+	+	+	+	+
10^{-3}	-	+	+	+	+	+
10^{-4}	-	-	-	+	+	+
10^{-5}	-	-	-	+	+	+
10^{-6}	-	-	-	-	-	+
10^{-7}	-	-	-	-	-	+
10^{-8}	-	-	-	-	-	-

24

Table 2. Detection of RT activity by Amp-RT in serum samples from HIV-1 infected individuals characterized by clinical stage, CD4+ lymphocyte counts in the peripheral blood and amount of p24 antigen in the serum.

No	Patient ID	p24 (pg/ml)	%CD4	CD4 count	Clinical Stage*	Amp-RT
1	69877	NEG	27	830	Al	NEG
2	82850	NEG	23	699	Al	P
3	69878	141	33	566	Al	P
4	77913	NEG	25	599	Al	P
5	111380	49	27	778	Al	NEG
6	111318	49	28	539	Al	NEG
7	85987	141	23	348	A2	P
8	66583	55	13	286	A2	P
9	62303	42	12	341	B2	P
10	71172	56	22	281	B2	NEG
11	71245	100	11	208	B2	P
12	77149	44	16	282	B2	P
13	86563	141	14	286	B2	P
14	66994	NEG	25	260	B2	NEG
15	57589	141	18	370	B2	P
16	21149	NEG	10	97	B3	P
17	70145	8	5	26	C3	NEG
18	84300	141	1	4	C3	P
19	111207	N	10	103	C3	P
20	67365	141	5	24	C3	P
21	96215	40	6	80	C3	P
22	120979	141	3	19	C3	P
23	20525	73	3	22	C3	P
24	736	103	8	50	C3	P
25	1837	158	3	6	C3	P
26	3125	150	5	12	C3	P

27	3369	33	1	1	C3	P
28	20577	85	6	123	C3	P
29	20525	73	3	22	C3	P
30	20577	85	6	123	C3	P
31	20709	158	10	42	C3	P
32	20876	80	7	57	C3	P
33	21156	124	14	106	C3	P
34	21196	24	8	48	C3	P
35	21684	45	4	25	C3	P
36	23505	64	4	19	C3	P
37	23507	15	3	52	C3	P
38	24462	149	6	41	C3	P
39	24705	149	5	128	C3	P
40	25238	98	5	13	C3	P
41	23209	71	5	10	C3	P
42	53416	NEG	14	81	C3	P

*A1 and A2 refer to asymptomatic, acute (primary) or persistent generalized lymphadenopathy with CD4+ lymphocyte counts of >500/ul or 200-499/ul, respectively. B2 and B3 refer to symptomatic conditions different from those in stage A or AIDS, with CD4+ lymphocyte counts of 200-499/ul and <200ul, respectively. C3 refers to AIDS, with CD4+ lymphocyte counts of <200/ul (29).

26

REFERENCES

1. Coffin, J.M. 1990. Retroviridae and their replication. In: Fields, B.N. et al., *Virology*, 2d Ed. Raven Press, New York.

2. Shinazi R.F. et al. 1992. Insights into HIV chemotherapy *AIDS Res. and Hum. Retroviruses* 8:963-990.

3. Willey, R.L. et al. 1988. In vitro mutagenesis identifies a region within the envelope gene for the human immunodeficiency virus that is critical for infectivity *J. Virol.* 62:139-147

4. Spira, T.J. et al. 1987. A micromethod for assaying the reverse transcriptase of HTLV-III/LAV *J. Clin. Microbiol.* 25:97-99

5. Eberle, J. et al. 1992. A new method of measuring reverse transcriptase activity by ELISA *J. Virol. Meth.* 40:347-356

6. Somogyi, P.A. et al. 1990. A solid phase reverse transcription micro-assay for the detection of human immunodeficiency virus and other retroviruses in cell culture supernatants *J. Virol. Meth.* 27:269-276

7. Cook, R.F. et al. 1991. A nonradioactive micro-assay for released reverse transcriptase activity of a lentivirus *Biotechniques* 13:380-386

8. Suzuki, K. et al. 1993. Detection of human immunodeficiency virus (HIV) by colorimetric assay for reverse transcriptase activity on magnetic beads *Biotechnol. Appl. Biochem.* 18:37-44

27

9.   Petry, H. et al. 1992. Isolation and characterization of a retrovirus from the fish genus *Xiphorus* *Virology* 188:785-792

10.   Phan-Thanh, L. et al. 1992. Porcine retrovirus: Optimal conditions for its biochemical detection *Arch. Virol.* 123:255-265

11.   De las Heras et al. 1991. Enzootic nasal tumour of goats: Demonstration of a type D-related retrovirus in nasal fluids and tumours *J. Gen. Virol.* 72:2533-2535

12.   Sano, K. et al. 1987. Antibody that inhibits human immunodeficiency virus reverse transcriptase and association with inability to isolate virus. *J. Clin. Microbiol.* 25:2415-2417

13.   Kageyama, S. et al. 1988. An improved method for the detection of HIV antigen in the blood of carriers *J. Virol. Meth.* 22:125-131

14.   Ho, D.D. et al. 1988. Quantitation of human immunodeficiency virus type 1 in the blood of infected persons *N. Eng. J. Med* 321:1621-1625

15.   Piatak, M. et al. 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR *Science* 259:1749-1754

16.   Mulder, J. et al. 1994. Rapid and simple PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma: Application to acute retroviral infection *J. Clin. Microbiol.* 32:292-300

17.   Innis, M.A. et al. 1990. *PCR Protocols: A Guide to Methods and Applications* Academic Press, San Diego, CA

18.   Pyra, H. et al. 1994. Ultrasensitive retrovirus detection by a reverse transcription assay based on product enhancement *Proc. Natl. Acad Sci.* USA 91:1544-1548

19.   Silver, J. et al. 1993. An RT-PCR assay for the enzyme activity of reverse transcriptase capable of detecting single virions *Nuc. Acids Res.* 21:3593-3594

20.   Silver, J. et al. 1994. Abstract from the Conference on Feasability of Genetic Technology to Close the HIV Window in Donor Screening, FDA, Maryland, September 26, 1994

21.   Wu, D.Y. et al. 1989. *Genomics* 4:560

22.   Barringer, K.J. et al. 1990. *Gene* 89:117

23.   Guatelli, J.C. et al. 1990. *Proc. Natl. Acad. Sci.* USA 87:1874

24.   Kwoh, D.Y. et al. 1989. *Proc. Natl. Acad. Sci.* USA 86:1173

25.   Lizardi, P.M. et al. 1988. *Bio/Technology* 6:1197

26.   McDougal, J.S. et al. 1985. Immunoassay for the detection and quantitation of infectious human retrovirus, lymphadenopathy-associated virus *J. Immunol. Meth.* 76:171-183

27.   Ou, C.Y. et al. 1988. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells *Science* 239:295-297

28.   Fishbein, D.B. et al. 1985. Unexplained lymphadenopathy in homosexual men- A longitudinal study *JAMA* 254:930-935

29.   Centers for Disease Control and Prevention. 1992. Revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents *Morbidity and Mortality Weekly Report* 41:1-19

30. Bavand, M. et al. 1989. The hepatitis B virus-associated reverse transcriptase is encoded by the viral pol gene *J. Virol.* 63:1019-1021

30

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: HENEINE, WALID
                 FOLKS, THOMAS M.
                 SWITZER, WILLIAM M.
                 YAMAMOTO, SHINJI

   (ii) TITLE OF INVENTION: METHODS FOR SENSITIVE DETECTION OF
        REVERSE TRANSCRIPTASE

  (iii) NUMBER OF SEQUENCES: 9

   (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: NEEDLE & ROSENBERG P.C.
        (B) STREET: 127 Peachtree Street, Suite 1200
        (C) CITY: Atlanta
        (D) STATE: Georgia
        (E) COUNTRY: USA
        (F) ZIP: 30303

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

   (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

 (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Spratt, Gwendolyn D.
        (B) REGISTRATION NUMBER: 36,016
        (C) REFERENCE/DOCKET NUMBER: 1414.628

   (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: 404/688-0770
        (B) TELEFAX: 404/688-9880

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CATTAGCCAT TTCAACCCAT   20

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTTCATGACA GGCCGATACA GAGG   24

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGCTCTCACC TTATCAAAAT CCAAT   25

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 374 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: RNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CAUUAGCCAU UUCAACCCAU GCGUUUGAGG AGAAGCGCUU UCUGAUAACC GGUGGUCUCC   60

CAUCAGGUUG UGCAGCGACC UCAAUGCUAA ACACUAUAAU GAAUAAUAUA AUAAUUAGGG   120

CGGGUUUGUA UCUCACGUAU AAAAAUUUUG AAUUUGAUGA UGUGAAGGUG UUGUCGUACG   180

GAGAUGAUCU CCUUGUGGCC ACAAAUUACC AAUUGGAUUU UGAUAAGGUG AGAGCAAGCC   240

UCGCAAAGAC AGGAUAUAAG AUAACUCCCG CUAACACAAC UUCUACCUUU CCUCUUAAUU   300

CGACGCUUGA AGACGUUGUC UUCUUAAAAA GAAAGUUUAA GAAAGAGGGC CCUCUGUAUC   360

GGCCUGUCAU GAAC   374

(2) INFORMATION FOR SEQ ID NO:5:

32

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 22 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTACCTAAT ACGACTCACT AT   22

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 49 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGTACCTAAT ACGACTCACT ATAGGGAGAC ATTAGCCATT TCAACCCAT   49

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 28 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATAATCCACC TATCCCAGTA GGAGAAAT   28

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 28 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTTGGTCCTT GTCTTATGTC CAGAATGC   28

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 41 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATCCTGGGAT TAAATAAAAT AGTAAGAATG TATAGCCCTA C   41

34

What is claimed is:

1.   A method for detecting the presence of a retrovirus in a biological sample comprising the steps of:

    a) contacting the biological sample with a suitable region of the encephalomyocarditis virus genome as an RNA template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample;

    b) amplifying the synthesized DNA; and

    c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

2.   The method of claim 1, wherein the complementary DNA primer is the oligonucleotide of SEQ ID NO:2.

3.   The method of claim 1, wherein the synthesized DNA is amplified by the polymerase chain reaction.

4.   The method of claim 3, wherein the conditions whereby the synthesized DNA is amplified comprise 30-40 cycles of heating the synthesized DNA and a primer pair to 93 to 96°C for 30 seconds to one minute, 53 to 56°C for 30 seconds to one minute and 70 to 74°C for 30 seconds to five minutes.

5.   The method of claim 4, wherein the conditions whereby the synthesized DNA is amplified comprise about 35 cycles of heating the synthesized DNA and a primer pair to about 95°C for one minute, about 55°C for one minute and about 72°C for one minute.

6.   The method of claim 5, wherein the primer pair consists of the oligonucleotide consisting essentially of SEQ ID NO:1 and the oligonucleotide consisting essentially of SEQ ID NO: 2.

7.   The method of claim 1, wherein the RNA template is the ribonucleotide of SEQ ID NO:4.

8.   The method of claim 1, wherein the amplification of the synthesized DNA is detected by Southern blot hybridization assay.

9.   The method of claim 8, wherein the synthesized DNA is detected in the Southern blot hybridization assay with a probe consisting essentially of the oligonucleotide of SEQ ID NO:3.

10.   A method of detecting the presence of a retrovirus in a biological sample comprising the steps of:

a) contacting the biological sample with an RNA template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample;

b) amplifying the synthesized DNA by the polymerase chain reaction method whereby the conditions for the amplification comprise 30-40 cycles of heating the synthesized DNA and a primer pair to 93 to 96°C for 30 seconds to one minute, 53 to 56°C for 30 seconds to one minute and 70 to 74°C for 30 seconds to five minutes; and

c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

11. The method of claim 10, wherein the RNA template is a region of the encephalomyocarditis virus genome.

12. The method of claim 11, wherein the RNA template is the ribonucleotide of SEQ ID NO:4.

13. The method of claim 10, wherein the conditions for the amplification of the DNA comprise about 35 cycles of heating the synthesized DNA and a primer pair to about 95°C for one minute, about 55°C for one minute and about 72°C for one minute.

14. The method of claim 13, wherein the primer pair consists of the oligonucleotide consisting essentially of SEQ ID NO:1 and the oligonucleotide consisting essentially of SEQ ID NO: 2.

15. The method of claim 10, wherein the amplification of the synthesized DNA is detected by Southern blot hybridization assay.

16. The method of claim 15, wherein the synthesized DNA is detected in the Southern blot hybridization assay with a probe consisting essentially of the oligonucleotide of SEQ ID NO:3.

17. A method for detecting the presence of a retrovirus in a biological sample comprising the steps of:

a) contacting the biological sample with a region of the encephalomyocarditis virus genome as an RNA template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample;

b) amplifying the synthesized DNA by the polymerase chain reaction method whereby the conditions for the amplification comprise 30-40 cycles of heating the synthesized DNA and a primer pair to 93 to 96°C for 30 seconds to one minute, 53 to 56°C for 30 seconds to one minute and 70 to 74°C for 30 seconds to five minutes; and

c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

18. The method of claim 17, wherein the complementary DNA primer is the oligonucleotide of SEQ ID NO:2.

19. The method of claim 17, wherein the amplification of the synthesized DNA is detected by Southern blot hybridization assay.

20. The method of claim 19, wherein the synthesized DNA is detected in the Southern blot hybridization assay with a probe consisting essentially of the oligonucleotide of SEQ ID NO:3.

21. The method of claim 17, wherein the RNA template is the ribonucleotide of SEQ ID NO:4.

22. The method of claim 21, wherein the conditions for the amplification of the DNA comprise about 35 cycles of heating the synthesized DNA and a primer pair to about 95°C for one minute, about 55°C for one minute and about 72°C for one minute.

23. The method of claim 22, wherein the primer pair consists of the oligonucleotide consisting essentially of SEQ ID NO:1 and the oligonucleotide consisting essentially of SEQ ID NO: 2.

24. A method of detecting the presence of a retrovirus in a biological sample comprising the steps of:

a) contacting the biological sample with a ribonucleotide of SEQ ID NO:4 and an oligonucleotide of SEQ ID NO:2 under conditions whereby the oligonucleotide and the ribonucleotide will anneal and a DNA strand will be synthesized as an extension from the oligonucleotide if reverse transcriptase is present in the sample;

b) amplifying the synthesized DNA by the polymerase chain reaction whereby the conditions of the amplification comprise about 35 cycles of heating the synthesized DNA and a primer pair consisting of the oligonucleotide consisting essentially of SEQ ID NO:1 and the oligonucleotide consisting essentially of SEQ ID NO:2 to about 95°C for one minute, about 55°C for one minute and about 72°C for one minute; and

c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

25. The method of claim 24, wherein the amplification of the synthesized DNA is detected by Southern blot hybridization assay.

26. The method of claim 25, wherein the synthesized DNA is detected in the Southern blot hybridization assay with a probe consisting essentially of the oligonucleotide of SEQ ID NO:3.

27. A kit for detecting the presence of a retrovirus in a biological sample, comprising a suitable region of the encephalomyocarditis virus genome as an RNA template and a complementary DNA primer for reverse transcriptase and a primer pair for polymerase chain reaction.

28. The kit of claim 27, wherein the complementary DNA primer is the oligonucleotide of SEQ ID NO:2.

29. The kit of claim 27, wherein the RNA template is the ribonucleotide of SEQ ID NO:4.

30. The kit of claim 27, wherein the primer pair consists of the oligonucleotide consisting essentially of SEQ ID NO:1 and the oligonucleotide consisting essentially of SEQ ID NO: 2.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12Q 1/48, C12Q 1/70, C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12Q, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPOQUE, PAJ, MEDLINE, BIOSIS, DERWENT BIOTECH ABSTRACTS, CA, SCISEARCH, PATENT CITATION INDEX, WPI, EMBL/SWISSPROT/ GENBANK/ DDBJ (STRAND)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A1, 9323560 (SCHÜPBACH, JÖRG), 25 November 1993 (25.11.93), page 21, line 5 - line 28; page 102 - page 108, claim 1	1-30
D,X	Medical Sciences, Volume 91, February 1994, Pyra Halina et al, "Ultrasensitive retrovirus detection by a reverse transcriptase assay based on product enhancement", page 1544 - page 1548, see whole document	1-30

[X] Further documents are listed in the continuation of Box C.   [X] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

13 May 1996

Date of mailing of the international search report

2 6. 06. 96

Name and mailing address of the ISA:

European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
Tel. (- 31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (31-70) 340-3016

Authorized officer

Patrick Andersson

Form PCT/ISA/210 (second sheet) (July 1992)

C (Continuation).   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim N:
A	Dialog Information Service, file 154, Medline, Dialog accession no. 08010946, Medline accession no. 92148946, Duke GM et al: "Sequence and and structural elements that contribute to efficient encephalomyocarditis virus RNA translation", J Virol (UNITED STATES) Mar 1992, 66 (3) p1602-9 + Emur1: ID=EMCPOLYP AC=M81861 -- ---------	2,6,9,14,16, 18,20,23,24, 25,26,28,30

International application No.

PCT/US 96/01257

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9323560	25/11/93	NONE	